Berberine induces pacemaker potential inhibition via cGMP-dependent ATP-sensitive K^+ channels by stimulating mu/delta opioid receptors in cultured interstitial cells of Cajal from mouse small intestine

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Received May 13, 2016; Accepted June 29, 2016

DOI: 10.3892/mmr.2016.5698

Abstract. Berberine is traditionally used to treat gastrointestinal (GI) motility disorders. The interstitial cells of Cajal (ICCs) are the pacemaker cells of the gastrointestinal tract, which are responsible for the production of gut movements. The present study aimed to investigate the effects of berberine on pacemaker potentials (PPs) in cultured ICC clusters from the mouse small intestine, and sought to identify the receptors involved and the underlying mechanisms of action. All experiments were performed on cultured ICCs, and a whole-cell patch-clamp configuration was used to record PPs from ICC clusters (current clamp mode). Under current clamp mode, berberine was shown to decrease the amplitude and frequency of PPs. However, these effects were suppressed by treatment with glibenclamide, a specific ATP-sensitive K^+ channel blocker. *Nor*-binaltorphimine dihydrochloride (a kappa opioid receptor antagonist) did not suppress berberine-induced PP inhibition, whereas ICI 174,864 (a delta opioid receptor antagonist) and CTOP (a mu opioid receptor antagonist) did suppress the inhibitory effects of berberine. Pretreatment with SQ-22536 (an adenylate cyclase inhibitor) or with KT-5720 (a protein kinase A inhibitor) did not suppress the effects of berberine; however, pretreatment with 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (a guanylate cyclase inhibitor) or KT-5823 [a protein kinase G (PKG) inhibitor] did. In addition, berberine stimulated cyclic guanosine monophosphate (cGMP) production in ICCs. These observations indicate that berberine may inhibit the pacemaker activity of ICC clusters via ATP-sensitive K^+ channels and the cGMP-PKG-dependent pathway by stimulating mu and delta opioid receptors. Therefore, berberine may provide a basis for the development of novel agents for the treatment of GI motility dysfunction.

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Key words: berberine, interstitial cells of Cajal, gastrointestinal tract, opioid receptor

Introduction

Gastrointestinal (GI) motility disorders comprise a family of digestive problems, which are caused by poorly understood neuromuscular dysfunction of the gut, and spastic or failed propulsion (motility) of food through the digestive system. GI motility patterns are highly integrated, require coordination between smooth muscle cells, and utilize regulatory inputs from neurons, and interstitial, endocrine and immune cells (1). The interstitial cells of Cajal (ICCs) are pacemaker cells, which are responsible for the production of gut movements alongside enteric neurons and smooth muscle cells (2-4). Transient receptor potential (TRP) melastatin 7 (TRPM7) is required for the pacemaking activity in murine small intestine (4) and $Ca2^+$-activated Cl^- channel [anoctamin 1 (ANO1)] is involved in slow wave current in ICCs (5). Therefore, TRPM7 and ANO1 are potentially promising novel targets for the pharmacological treatment of GI motility disorders. Several neurotransmitters and hormones may affect ICC activity, thus modulating gut motility (5). For example, Jatrorrhizine is one of the major protoberberine alkaloids isolated from manymedicinal plants, including *Berberis aristata* and *Coptis chinensis*. Traditional oriental medicine uses the extracts of these plants for the treatment of gastroenteritis and diarrhea (6). Numerous traditional oriental medicines have been used to treat GI motility disorders (6).

Berberine is an isoquinoline alkaloid purified from *Berberis* sp., which exerts various biochemical and pharmacological effects (7,8). In addition, it possesses significant antimicrobial activity towards several organisms, including bacteria, fungi, protozoans and helminths (9,10). Berberine is traditionally used as an antidiarrheal agent (11,12), and this effect is thought to be dependent on its antibacterial activity (12,13). Furthermore, berberine has been reported to inhibit acetylcholine- or Ba^{2+}-induced contraction of guinea pig ileum and colonic smooth muscle (12). However, to the best of our knowledge, the effects of berberine on ICCs have

not been previously investigated. Therefore, the present study aimed to investigate the effects of berberine on the pacemaker potentials (PPs) of cultured ICC clusters from the murine small intestine.

Materials and methods

Ethics. Animal care and experiments were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Busan, South Korea; approval no. PNU-2015-1036) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23; 1996 revision).

Preparation of cells and cell cultures. BALB/c mice (age, 3-7 days weight, 1.9-2.2 g; Samtako Bio Korea Inc., Osan-si, Korea) were anesthetized with 0.1% ether and euthanized by cervical dislocation. They were maintained under controlled conditions (temperature, 21±3˚C; humidity 50±6%; 12 h light/dark cycles) and were allowed free access to food and water. Small intestines were excised from 1 cm below the pyloric ring to the cecum, and were opened along the mesenteric border. Luminal contents were removed using Krebs Ringer bicarbonate solution, tissues were pinned to the bases of Sylgard dishes, and mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle, which consist of circular and longitudinal muscles, were equilibrated for 30 min in Ca^{2+}-free Hank's solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose, and 11 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH 7.4. Cells were then dispersed in an enzyme solution containing collagenase (1.3 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA), bovine serum albumin (2 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), trypsin inhibitor (2 mg/ml; Sigma-Aldrich; Merck Millipore) and ATP (0.27 mg/ml), and were plated onto sterile glass coverslips coated with murine collagen (2.5 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA) in 35 mm culture dishes. Subsequently, the cells were cultured at 37˚C in an atmosphere containing 95% oxygen/5% carbon dioxide in smooth muscle growth medium (Clonetics™; Lonza, Basel, Switzerland) supplemented with 2% antibiotics/antimycotics (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and murine stem cell factor (5 ng/ml; Sigma-Aldrich; Merck Millipore). All experiments on ICC clusters were performed following 12 h of culture. ICCs were identified immunologically by incubating with a phycoerythrin-conjugated rat anti-mouse c-Kit monoclonal antibody (cat. no. 12-1172; eBioscience, Inc., San Diego, CA, USA), at a dilution of 1:50 for 20 min at 37˚C. Since the ICC morphology differed from the other cell types in culture, identification was possible under a phase contrast microscope following incubation with the anti-c-Kit antibody.

Patch clamp experiments. Physiological salt solution [5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES (adjusted to pH 7.4 with NaOH)] was used to bathe cultured ICC clusters (Na^+-Tyrode). The pipette solution used to examine pacemaker activity consisted of the following reagents: 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM dipotassium ATP, 0.1 mM sodium guanosine-5'-triphosphate, 2.5 mM creatine phosphate disodium, 5 mM HEPES and 0.1 mM ethylene glycol tetra-acetic acid (adjusted to pH 7.2 with KOH). Patch clamp techniques were conducted in whole-cell configuration to record potentials (i.e., current clamp mode) from cultured ICCs using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, Inc., Foster, CA, USA). Command pulses were applied using an IBM-compatible personal computer (Compaq Computer Corporation, Houston, TX, USA) and pClamp software (versions 6.1 and 10.0; Axon Instruments, Inc.). Data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and/or a pen recorder (Gould 2200; Gould Instruments, Inc., Valley View, OH, USA). Results were analyzed using pClamp and Origin software (version 6.0; MicroCal, Northampton, MA, USA). All experiments were performed at 30-33˚C.

Cyclic guanosine monophosphate (cGMP) assay. ICCs were preincubated with 100 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck Millipore) for 30 min at 37˚C to inhibit cGMP degradation, and were then incubated with berberine (50 μM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 10 min. Following homogenization in a buffer containing 4 mM ethylenediaminetetraacetic acid to prevent degradation of enzymatic cGMP, homogenates were heated for 5 min in a boiling water bath to coagulate proteins, and were then centrifuged at 3,950 x *g* for 5 min at 4˚C. The supernatants subsequently obtained were transferred to fresh tubes and were stored at 4˚C. Samples were assayed for cGMP using cGMP enzyme-linked immunosorbent assay kits (Enzo Life Sciences, Inc., Farmingdale, NY, USA). These assays were conducted according to the manufacturer's protocol.

Drugs. Opioid receptor antagonists were purchased from Tocris Bioscience (Minneapolis, MN, USA); all other drugs were obtained from Sigma-Aldrich (Merck Millipore). Stock solutions were prepared and stored in accordance with the manufacturers' protocols. Chemicals were dissolved in Na^+-Tyrode solution to obtain their final concentrations immediately prior to use. Berberine was dissolved in methanol to produce a 50 mmol/l stock solution, which was subsequently added to the bathing solution at a final concentration of 50 μM on the day of the experiment for 5 min. The final concentration of methanol in the bathing solution was <0.1% and preliminary experiments confirmed that this concentration of methanol did not affect results. In addition, glibenclamide (Sigma-Aldrich; Merck Millipore) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck Millipore) to produce a 10 mmol/l stock solution, which was added to the bathing solution at a final concentration of 10 μM on the day of the experiment for 5 min. Next, *nor*-Binaltorphimine dihydrochloride (Tocris Bioscience) was dissolved in distilled water to produce a 25 mmol/l stock solution, which was added to the bathing solution at a final concentration of 100 nM on the day of the experiment. ICI 174,864 (Tocris Bioscience) and CTOP (Tocris Bioscience) was directly added to the bathing solution at a concentration of 20 μM and 10 μM on the day of the experiment. Both SQ-22536 (Sigma-Aldrich; Merck Millipore) and ODQ (Sigma-Aldrich; Merck Millipore) were dissolved in DMSO to produce a 100 mmol/l stock solution,

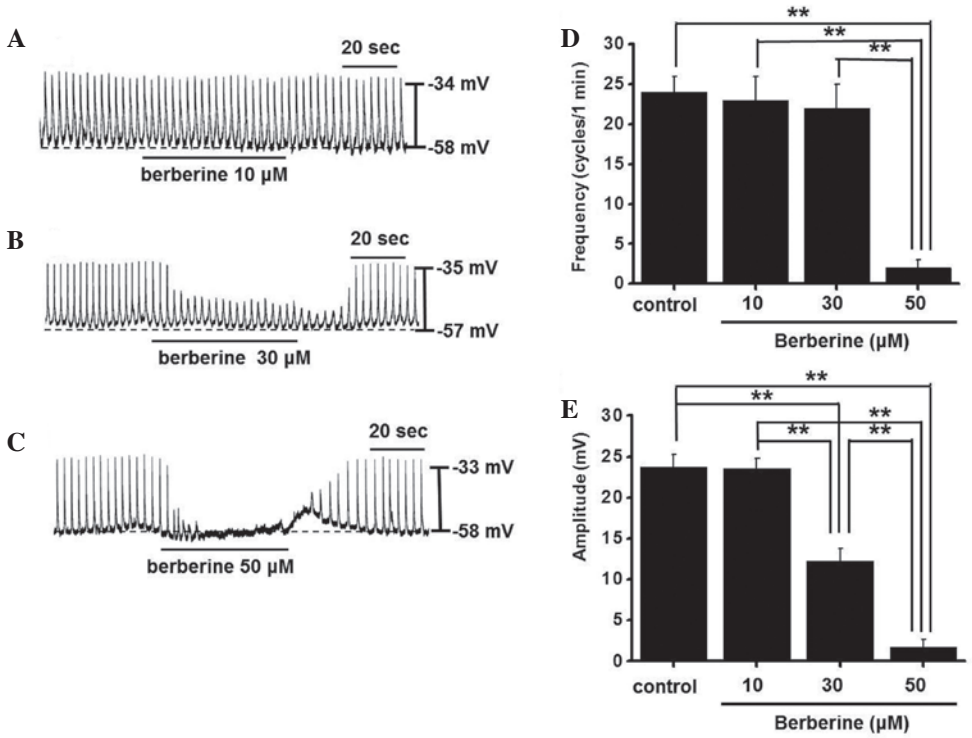

Figure 1. Effects of berberine on the pacemaker potentials (PPs) of cultured interstitial cells of Cajal (ICC) clusters from murine small intestine. (A-C) PPs of ICCs exposed to berberine (10, 30 and 50 μM) in current-clamp mode (I=0). Berberine decreased the amplitude and frequency of PPs in a concentration-dependent manner. (D and E) Graphs summarizing responses to berberine. Results are presented as the mean ± standard error of the mean. **P<0.05.

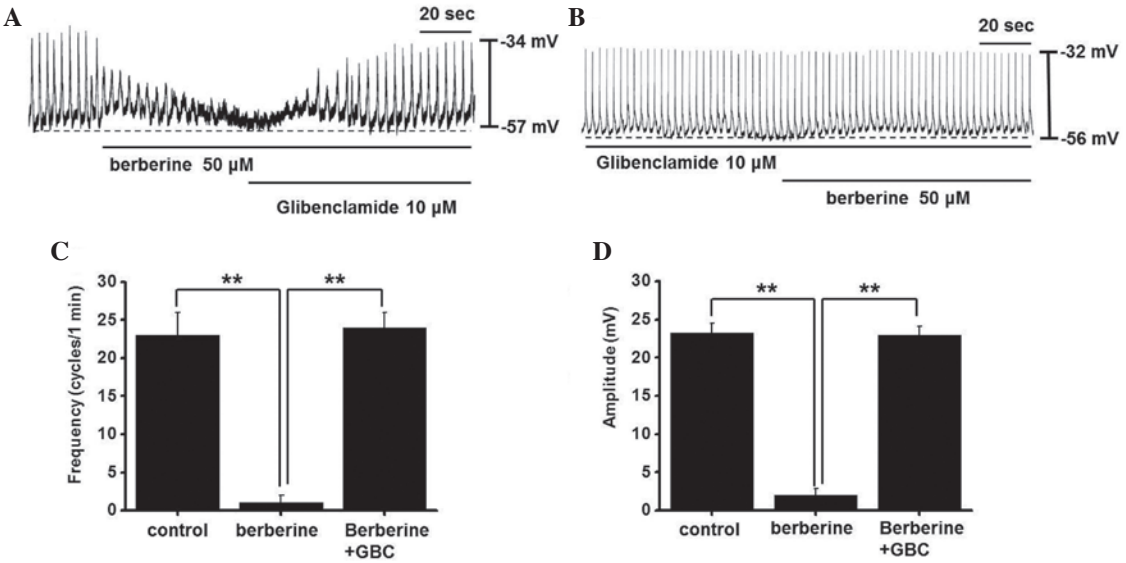

Figure 2. Effects of glibenclamide (GBC) and berberine on the pacemaker potentials (PPs) of cultured interstitial cells of Cajal clusters from murine small intestine. (A) The decreased amplitude and frequency of PPs induced by berberine were prevented by GBC (10 μM). (B) Treatment with GBC alone did not affect PPs. Following GBC pretreatment, berberine did not inhibit PPs. (C and D) Effects of berberine and GBC on the frequency and amplitude of PPs. Results are presented as the mean ± standard error of the mean. **P<0.05.

which was added to the bathing solution at a final concentration of 100 μM on the day of the experiment for 5 min. Also, KT-5720 (Sigma-Aldrich; Merck Millipore) and KT-5823 Sigma-Aldrich; Merck Millipore) were dissolved in DMSO to produce 1 mmol/l stock solutions, which were added to the bath solution at a final concentration of 1 μM on the day of the experiment for 5 min.

Statistical analysis. The data are presented as the mean ± standard error of the mean. Student's t-test and one-way analysis of variance followed by Bonferroni's post-hoc test were used to determine significance. P<0.05 was considered to indicate a statistically significant difference. For statistical analyses, Prism version 5.0 (GraphPad, Software Inc., La Jolla, CA, USA) and Origin version 8.0 (OriginLab Corporation,

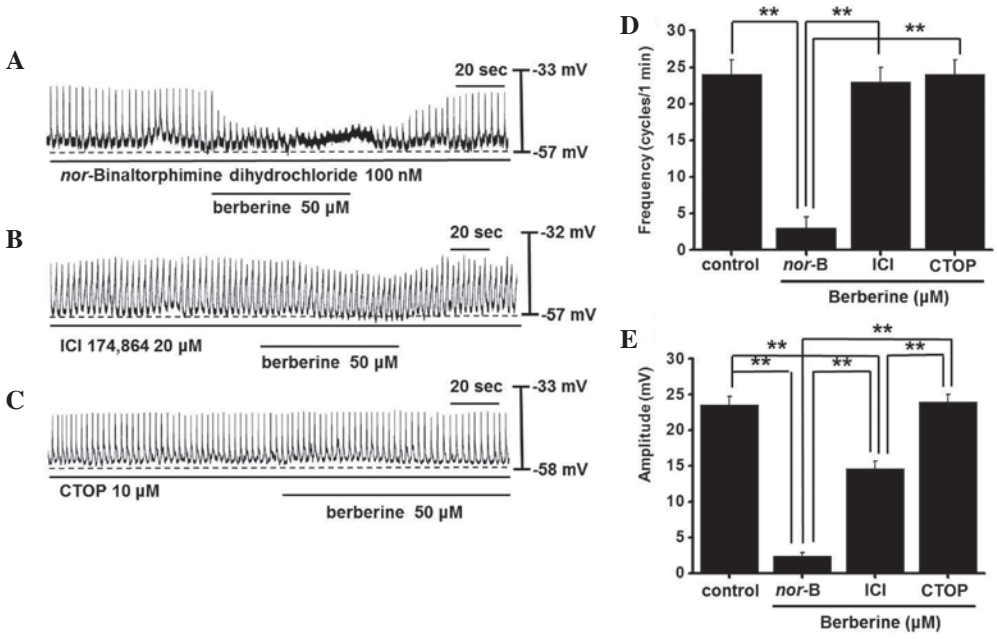

Figure 3. Effects of opioid receptor antagonists on berberine-induced PP inhibition in cultured interstitial cells of Cajal clusters. (A) *Nor*-binaltorphimine dihydrochloride (*nor*-B; a kappa opioid receptor antagonist) did not inhibit berberine-induced responses, whereas (B) ICI 174,864 (ICI; a delta opioid receptor antagonist) or (C) CTOP (a mu opioid receptor antagonist) suppressed these responses. (D and E) Effects of berberine and opioid receptor antagonists on the frequency and amplitude of PPs. Results are presented as the mean ± standard error of the mean. $^{**}P<0.05$.

Northampton, MA, USA) were used. The n values reported in the text refer to the number of cells used in patch clamp experiments. Experiments were repeated 6-8 times.

Results

Effects of berberine on PPs in cultured ICC clusters. The ICCs generated PPs under whole cell patch current clamp mode (I=0) (Fig. 1) at a mean frequency of 24.1±2.1 cycles/min and a mean amplitude of 23.8±1.5 mV. Treatment with berberine (10-50 μM) inhibited PPs and decreased their amplitudes in a concentration-dependent manner (Fig. 1A-C). In the presence of berberine, mean PP frequencies were 23.1±3.2 cycles/min at 10 μM, 22.2±3.1 cycles/min at 30 μM, and 2.2±1.3 cycles/min at 50 μM (Fig. 1D, n=18), whereas mean amplitudes were 23.5±1.4 mV at 10 μM, 12.3±1.5 mV at 30 μM, and 1.8±0.9 mV at 50 μM (Fig. 1E, n=18). These results suggest that berberine may dose-dependently inhibit the PPs of ICCs.

Berberine activates ATP-sensitive K^+ channels in cultured ICC clusters. In our previous study, pinacidil (an ATP-sensitive K^+ channel opener) decreased the frequency and amplitude of PPs, and these pinacidil-induced effects were reversed by treatment with glibenclamide (an ATP-sensitive K^+ channel blocker) (14). In the present study, berberine-induced ICC PP inhibition was suppressed by glibenclamide (Fig. 2A), and following glibenclamide pretreatment, berberine exerted no effects on PPs (Fig. 2B). The effects of berberine and glibenclamide on PPs are presented in Fig. 2C and D. These results suggest that berberine may inhibit PPs via ATP-sensitive K^+ channels in ICCs.

Identification of berberine receptor subtypes in cultured ICC clusters. To investigate the association between berberine and its receptors in cultured ICCs, the opioid receptors were investigated, since they are known to be involved in the regulation of GI motility (15-17). There are three major classes of opioid receptor (mu, delta and kappa) in the GI tract (15-17). To identify the opioid receptor subtypes associated with the effects of berberine, ICCs were pretreated with opioid receptor antagonists, followed by treatment with berberine. *Nor*-binaltorphimine dihydrochloride (a kappa opioid receptor antagonist), ICI 174,864 (a delta opioid receptor antagonist) and CTOP (a mu opioid receptor antagonist) were used to pretreat the cells for 5 min, followed by berberine treatment (50 μM) (Fig. 3). Treatment with these opioid receptor antagonists alone had no effect on PPs; however, pretreatment with ICI 174,864 or CTOP suppressed berberine-induced PP inhibition (Fig. 3B and C). In the presence of ICI 174,864 or CTOP, the mean amplitudes of berberine-induced PPs were 14.6±1.1 and 24.1±1.2 mV, respectively (n=6; Fig. 3E). Conversely, pretreatment with *nor*-binaltorphimine dihydrochloride did not suppress berberine-induced PP inhibition (Fig. 3A). In the presence of *nor*-binaltorphimine dihydrochloride, the mean frequency and amplitude of berberine-induced PPs were 3.1±1.4 cycles/min and 2.4±0.5 mV, respectively (n=6; Fig. 3D and E). These results suggest that berberine may affect ICCs via mu and delta opioid receptors.

Association of guanylate cyclase and protein kinase G (PKG) with berberine-induced PP inhibition. To determine whether berberine-induced PP inhibition is mediated by a cyclic nucleotide-dependent pathway, an adenylate cyclase inhibitor (SQ-22536) and guanylate cyclase inhibitor (1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one; ODQ) were used to treat the ICC clusters. Preincubation with SQ-22536 (100 μM) alone for 5 min had no effect on PPs, and in the presence of SQ-22536, berberine (50 μM) still inhibited PPs (Fig. 4A). However, ODQ (100 μM) suppressed berberine-induced PP inhibition (Fig. 4B).

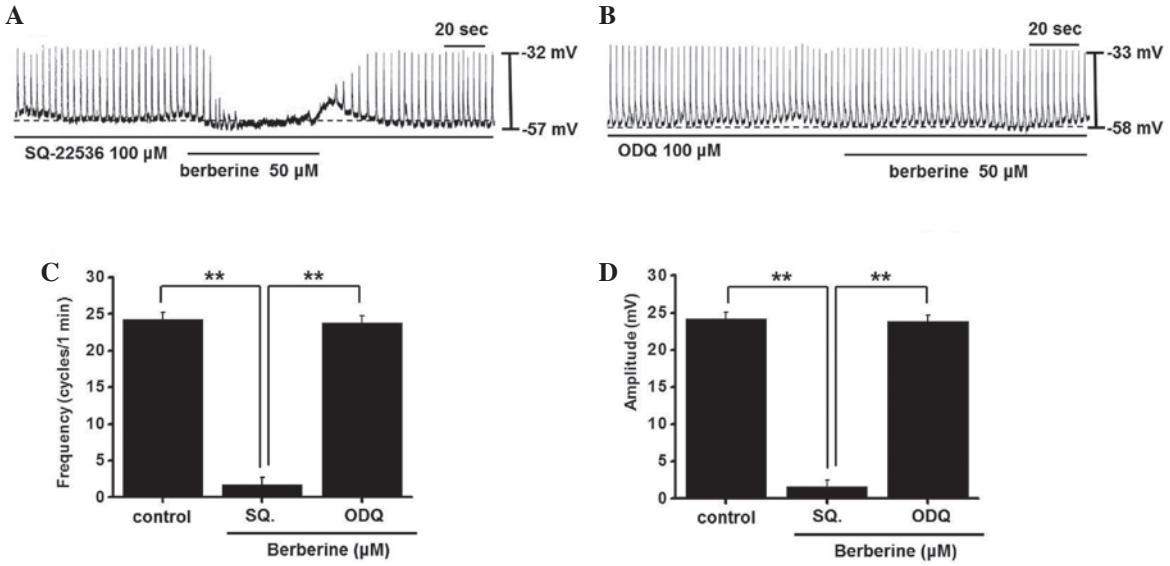

Figure 4. Effects of SQ-22536 (SQ; an adenylate cyclase inhibitor) and 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; a guanylate cyclase inhibitor) on berberine-induced pacemaker potential (PP) inhibition in cultured interstitial cells of Cajal clusters. (A) SQ (100 μM) had no effect on berberine-induced PP inhibition. However, (B) ODQ (100 μM) suppressed berberine-induced PP. (C and D) Effects of berberine and cyclase inhibitors on the frequency and amplitude of PPs. Results are presented as the mean ± standard error of the mean. **P<0.05.

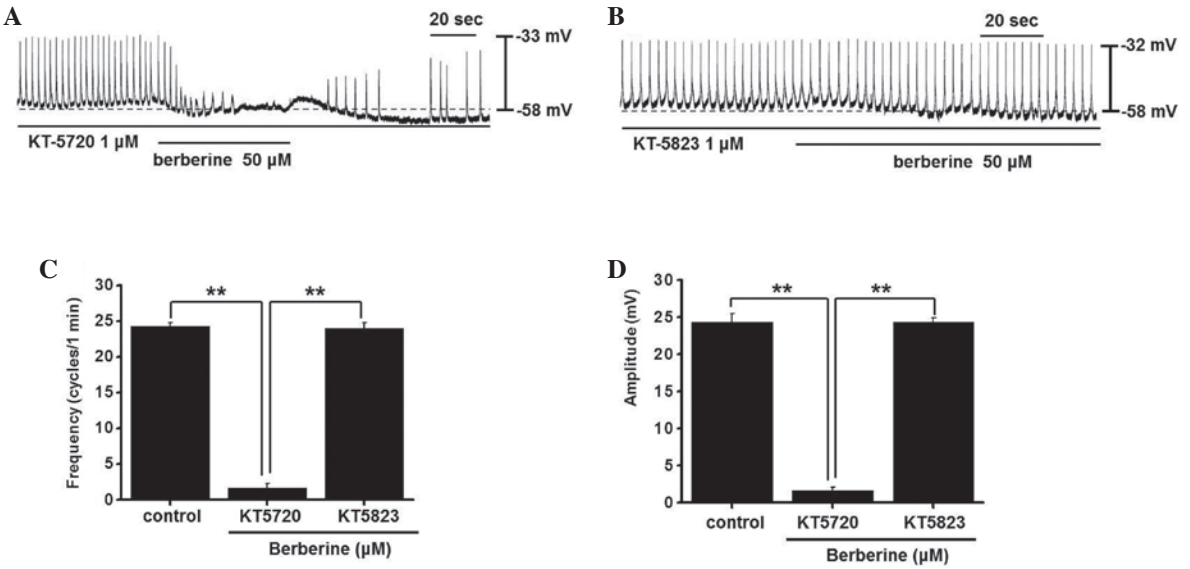

Figure 5. Effects of KT-5720 (a protein kinase A inhibitor) and KT-5823 (a protein kinase G inhibitor) on berberine-induced pacemaker potential (PP) inhibition in cultured interstitial cells of Cajal clusters. (A) KT-5720 (1 μM) had no effect on berberine-induced PP inhibition. However, (B) KT-5823 (1 μM) suppressed the inhibitory effects of berberine. (C and D) Effects of berberine and protein kinase inhibitors on the frequency and amplitude of PPs. Results are presented as the mean ± standard error of the mean. **P<0.05.

In the presence of SQ-22536, the mean frequency and amplitude of berberine-induced PPs were 1.8±0.9 cycles/min and 1.6±0.9 mV, respectively (n=6; Fig. 4C and D); the ODQ corresponding values were 23.7±1.0 cycles/min and 23.8±0.8 mV, respectively (n=6; Fig. 4C and D). In addition, the effects of a protein kinase A inhibitor (KT-5720) and a PKG inhibitor (KT-5823) were detected. Preincubation of ICCs with KT-5720 or KT-5823 alone had no effect on PPs. Furthermore, in the presence of KT-5720 (1 μM), berberine (50 μM) inhibited PPs (Fig. 5A); however, preincubation with KT-5823 (1 μM) suppressed berberine-induced PP inhibition (Fig. 5B). In addition, intracellular cGMP contents were measured under basal and berberine-stimulated conditions, and berberine was revealed to stimulate cGMP production [Fig. 6; control (12.1±0.6 pmol/mg protein) vs. berberine (14.3±0.9 pmol/mg protein)]. These results suggest that cGMP and PKG may have roles in berberine-induced PP inhibition.

Discussion

The present study investigated the effects of berberine on PPs in cultured ICC clusters from the mouse small intestine,

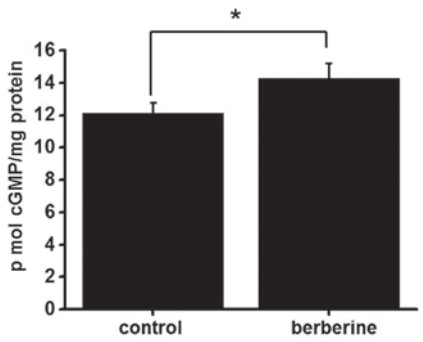

Figure 6. Association of cyclic guanosine monophosphate (cGMP) production with berberine-induced pacemaker potential inhibition in cultured interstitial cells of Cajal (ICC) clusters. Preincubation of ICCs with berberine significantly stimulated cGMP production. Results are presented as the mean ± standard error of the mean. *P<0.05.

and sought to identify the receptors involved and the underlying mechanisms of action. The results demonstrated that berberine may inhibit the pacemaker activity of ICC clusters via ATP-sensitive K^+ channels and the cGMP-PKG-dependent pathway by stimulating mu and delta opioid receptors. These findings suggested that berberine offers a basis for the development of novel treatments for GI motility dysfunction.

Berberine is an isoquinoline alkaloid, which is present in numerous plant species, including *Coptis* sp. and *Berberis* sp. Berberine is component in traditional Chinese medicines, and is used to treat diarrhea and gastroenteritis. Berberine possesses antimicrobial, antimotility and antisecretory properties (18), and berberine and its derivatives exert potent analgesic (19), anti-inflammatory (20) and anticancer (21) activities in the GI tract. Furthermore, berberine reportedly exerts potential therapeutic effects on diabetes (22), hyperlipidemia (23), cardiovascular diseases (24) and central nervous system disorders (25). In addition, berberine affects GI motility, since it is able to significantly reduce smooth muscle contractility and intestinal motility, and may delay intestinal transit times in rodents, as determined by intestinal myoelectric activity experiments (16,26). The inhibitory effects of berberine may be explained by the upregulation of somatostatin and glucagon-like peptide-1 levels, and the downregulation of motilin and gastrin levels (16,26). Berberine has also been reported to block muscarinic receptors in guinea pig longitudinal muscle isolated from the ileum, and therefore may reduce intestinal motility (27). Furthermore, berberine exerts dopamine D_2 receptor antagonist and 5-HT_{1A} receptor agonist properties, and exhibits significant potential as a therapeutic agent for the treatment of functional dyspepsia (28). Berberine has also been reported to exert stimulatory effects in low contractile states, and inhibitory effects in high contractile states on rat jejunal segments (29). Berberine-induced GI motility regulation could also be explained by the endogenous opioid system, since opioid receptors are associated with the regulation of GI motility, and inhibition of the opioid system suppresses the inhibitory effects of berberine on intestinal activity (16,30).

ICCs act as gut pacemaker cells, and previous studies have suggested that ICC networks coordinate peristaltic movement (4,5). Due to the important roles of ICCs, hormones and neurotransmitters that affect ICC activity are considered to exert a significant influence on gut motility (2).

The present study demonstrated that berberine may decrease the frequency and amplitude of PPs in a dose-dependent manner in ICCs (Fig. 1), and that these effects may be mediated in a cGMP (Fig. 4) and PKG-dependent manner (Fig. 5) via ATP-sensitive K^+ channels (Fig. 2). In addition, mu and delta opioid receptors were revealed to be mechanistically associated with the effects of berberine (Fig. 3). Opioids and opiates affect various GI functions, including motility, secretion, and the transport of electrolytes and fluids, by activating the three major classes of opioid receptor (delta, kappa and mu) (15,31,32). Mu and kappa opioid receptor immunoreactivities have been described in the enteric neurons of rats and guinea pigs, and in the porcine GI tract, whereas delta opioid receptor immunoreactivity has been reported in porcine ileum. In addition, mu and kappa opioid receptors are localized in ICCs of the myenteric plexus and deep muscular plexus in rats (33). The various effects of opioids and opiates are dependent on opioid receptor activation. These receptors belong to the seven transmembrane G-protein-coupled receptor superfamily (15,31,32). In the present study, mu and delta opioid receptors were shown to be involved in berberine-induced PP inhibition (Fig. 3). Future studies should focus on investigating the immunohistochemical expression level of opioid receptors in murine GI tract.

The results of the present study indicated that berberine inhibits ICC function. However, since GI motility patterns are highly integrated, and require coordination between smooth muscle cells and regulatory inputs from interstitial cells, neurons, and endocrine and immune cells (1), further investigations regarding the effects of berberine on smooth muscle cells, the enteric nervous system, and endocrine and immune cells *in vitro*, and on GI motility *in vivo*. are required.

In conclusion, the results of the present study suggested that berberine reduces the amplitude and frequency of ICC PPs in a cGMP-, and PKG-dependent manner via ATP-sensitive K^+ channels by stimulating mu and delta opioid receptors. These findings indicated that berberine may be a potential drug development candidate for the treatment of GI motility disorders, including spasms, pain and transit disturbances.

Acknowledgements

The present study was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (grant no. 2014R1A5A2009936).

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